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Atty. Docket No. SYP-101DV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): Jindal et al.

SERIAL NO.: Divisional of U.S.S.N. 09/267,993 GROUP NO.: 1627 (prior appl'n)

FILED: December 5, 2001 EXAMINER: B. Celsa (prior appl'n)

TITLE: HIGH SPEED, AUTOMATED, CONTINUOUS FLOW, MULTIDIMENSIONAL MOLECULAR SELECTION AND ANALYSIS

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PRELIMINARY AMENDMENT

Sir:

Please enter this Preliminary Amendment for the above-identified divisional patent application prior to calculating the filing fee. Claims 1-48 are cancelled without prejudice and new claims 49-66 are introduced herein. Accordingly, after entry of this amendment, claims 49-66 will be pending for examination.

AMENDMENTS

To the Specification

Please amend paragraphs of the specification to read as follows. (A marked up version of the amended paragraphs is attached at the end of this Preliminary Amendment.)

Page 1, lines 9-12

RELATED APPLICATIONS

This application is a divisional of application Serial No. 09/267,993, filed on March 12, 1999, which is a continuation of Serial No. 08/670,670, filed on June 26, 1996, now abandoned, which claims the benefit of Serial No. 60/000,518, filed on June 26, 1995, now expired, the entire disclosures of which are incorporated by reference herein.

Page 56, line 15 to page 57, line 6

Monoclonal antibody (mAb) chosen (mouse IgG2a, clone 3E-7, Boehringer Mannheim, Indianapolis, IN) was raised against human β-endorphin and recognizes the amino terminus of β-endorphin, YGGFL (SEQ ID NO: 1). The purchased mAb (280mg resuspended in 1ml H_2O) was passed over an XL cartridge (2.1 x 30mm) consisting of protein-G coupled to POROS™ perfusion chromatographic media (PerSeptive Biosystems, Framingham, MA) by making 10 x 100ml injections on a BioCAD™ 20 Workstation (PerSeptive Biosystems, Framingham, MA). Protein-G binds to the Fc region of antibodies with high affinity. The mAb was subsequently cross-linked to the protein G using the standard methods and materials provided with the XL column. In brief, this consisted of passing 14 ml of cross-linking solution (100 mM triethanolamine, pH 8.5, 7.8 mg/ml dimethyl pimelimidate (DMP)) over the column at a flow rate of 0.5 ml/min. The cross-linking reagent was quenched by subsequent injection of 2 ml of 100 mM monoethanolamine, pH 9.0. The sensor cartridge was washed with PBS, pH 7.4 at 0.5 ml/min for 2 min followed by a further injection of 2 ml of quench solution and washed as above. The antibody was efficiently immobilized to the column as demonstrated by the lack of reactivity to coomassie stain upon SDS-PAGE of the flow through. A second XL column (without antibody) was treated with cross-linking reagents and washed as for the affinity column for use as a "control column".

Page 57, lines 15-22

To investigate the binding of the immobilized antibody to its epitope YGGFL (SEQ ID NO: 1), the synthetic peptide YGGFL (SEQ ID NO: 1) (Sigma Chemical Co., St. Louis, MO; 1 mg/ml in PBS, pH 7.4) was injected onto the affinity column (flow rate 0.2 ml/min). After washings with 10 column volumes (CV) of PBS, pH 7.4, the bound peptide was eluted from the affinity column with 12 mM HCl, directly onto the vydac C-18 column. This reversed phase column was then developed with a gradient of acetonitrile (4% ACN/12 mM HCl to 80% ACN/6 mM HCl over 18 min; flow rate 1 ml/min). Both columns were re-equilibrated in their respective starting buffers prior to the next injection.

Page 58, lines 16-24

As a first step, whether the mAb immobilized onto the XL cartridge retained its ability to bind the peptide YGGFL (SEQ ID NO: 1) was investigated. The peptide solution (20 nmol) was injected onto the affinity column and then unbound peptide was removed by washing the column with PBS, pH, 7.4. Peptides affinity captured by the antibody was eluted from the " target column" directly onto the C-18 column for resolution. The amount of the peptide recovered, as calculated from the peak area, was approximately 1.2 nmoles. The theoretical capacity based on the quantity of mAb loaded onto the affinity column was 3.8 nmol demonstrating that approximately 25% of the binding sites of the mAb are available in a conformationally active form.

Page 59, lines 2-14

The capacity of the column was examined by injecting increasing larger quantities of the peptide (YGGFL) (SEQ ID NO: 1) utilizing the loading template of the BioCAD™ Workstation. The amount of the bound peptide (as calculated from the peak height) reached saturation at about 1.2 nmoles. Interestingly, the amount of peptide bound to the antibody was independent of the flow used to inject the peptide. Increasing the flow rate from 0.2ml/min to 5 ml/min did not affect the recovery. This result suggests a rapid interaction of the peptide with the antibody during the loading process when a perfusive packing material and elution conditions are used. The EC_{50} value (50% of the saturating amount) for the peptide is approximately 30 nmols. This value correlates well with the affinity constants determined previously for binding of YGGFL (SEQ ID NO: 1) peptide to 3E-7 by competitive radiolabelled binding assays (See Lam *et. al.*, *Biorganic Med. Chem. Letts. 3:*419-424 (1993).

Page 59, line 21 to page 60, line 8

Using the conditions established for purified YGGFL (SEQ ID NO: 1) binding to the mAb column, the XXXFL library was screened for moieties recognized by the target immobilized mAb affinity column. The library (containing 2.8nmols of each peptide) was loaded

onto the "target column" , the unbound material was washed with 10 Column volumes of PBS, pH 7.4. Finally, the affinity bound material was eluted directly onto the vydac C-18 column with 12 mM HCl. Elution of the C-18 column (with a 4-80% ACN gradient as described above) revealed approximately 10-12 resolvable peaks. The elution profile shows that, one of the peaks observed exhibits a retention time comparable to that observed for pure YGGFL (SEQ ID NO: 1). Using the chosen wash conditions (10 CV) for the immunoaffinity column, we were able to selectively discriminate a single moiety from a library with potentially greater than 5800 individual peptides. The identity of isolated YGGFL (SEQ ID NO: 1) was confirmed by mass spectrometry and peptide sequencing.

Page 60, lines 11-16

In order to confirm the specificity of the target immobilized affinity column for YGGFL (SEQ ID NO: 1), samples of the purchased peptide and the library (XXXFL) were analyzed in a parallel experiment using the control column prepared in Experiment 1. The chromatogram was identical except for the peak corresponding to YGGFL (SEQ ID NO: 1). In a parallel experiment, a peptide with the sequence YEYFL (SEQ ID NO: 2) (a known non-binder to the mAb was not retained by the affinity column.

Page 62, line 20 to page 63, line 19

Furthermore, increasing the number of column volumes used to wash column 1 prior to elution onto column 2 resulted in a decrease in the subsequent recovery of these molecules, presumably due to dissociation of these agents from the LPS while on column 1. The number of wash volumes required to reduce the peak for these agents correlated with the reported affinities of these agents for LPS; PmxB (Kd 0.4 mM) was reduced 50% after 42 CVs washing of column 1 while pentamidine (Kd 100 nM) required a higher number of washes for a similar reduction (46 CVs). Using this methodology we could rapidly examine which peaks from the library were capable of binding to LPS with the highest affinity since, upon exposure to higher numbers of wash volumes only the peptides with higher affinity for the target were retained. The relative reduction in the peak area (or height) for each peak eluted from the reverse phase column when plotted against the number of CVs used to wash column 1 could be used to distinguish the peaks

with higher affinity since these showed a lower rate of reduction under the same conditions. Three such peaks from the XXXFL library and 3 peaks from the CXXXC library were further purified and characterized by MS and peptide sequencing. Results from the sequencing data for the XXXFL candidates suggested the structures RRRFL (SEQ ID NO: 3), RRKFL (SEQ ID NO: 4) or KKRFL (SEQ ID NO: 5). The latter peptide has been synthesized and demonstrated to bind to LPS using the same paradigm above. However, while this peptide is displaced by Pentamidine in a competition experiment, its affinity for LPS is much lower than the latter molecule. The other peptides will be synthesized and studied in a similar manner although another approach being considered is that a sublibrary of the format ZZZFL (where Z represents either R or K) be screened to identify the member with the highest affinity. Candidates purified from the cyclic library are still awaiting sequencing.

Page 66, line 25 to page 67, line 6

Figure 8 shows the results of this data. From this data we 'named' the peptide HHRSY (SEQ ID NO: 6) as being composed of the amino acids that showed the greatest increase in AA% when the column lacked sugar relative to its AA% in the presence of sugar. Synthesis of this peptide and characterization of its ability to bind revealed that it was capable of binding specifically to Con A immobilized on the column with little binding to the control column. Furthermore, this peptide could be displaced somewhat by inclusion of a high concentration of the competing sugar ligand. These data suggest that it is possible to identify ligands for a specific site on a molecule by using this bimolecular approach to screening.

Page 73, lines 2-11

2) Direct synthesis of peptides on POROS™: The 19-mer peptide (TVTEKPEVIDASELTPAVT) (SEQ ID NO: 7) selected from protein A and G digests was directly synthesized on 20 μm amine-functionalized ™ particles by standard FMOC chemistry. About 700 mg of resin was dry packed into a 4.6 mm D x 50 mm L PEEK column equipped with 2 μm frits. The packed column was attached with appropriate adapters to a PerSeptive Biosystems 9050 plus continuous flow peptide synthesizer. Upon completion of synthesis, the

resin was dried and deprotected using 95% TFA/5% triisopropylsilane for 24hrs. The final peptide-support conjugate was packed using POROS™ Self Pack® column packing device at flow rate 10 ml/min onto 4.6 mm D x 50 mm L column for evaluation as affinity supports.

Page 74, lines 1-11

3) Purification of IgG (whole and Fc fragment) on POROS™-peptide (TVTEKPEVIDASELTPAVT) (SEQ ID NO: 7) column: The 19-mer peptide column (4.6 mm D x 50 mm L) was equilibrated with 20 CVs of equilibration buffer, 20 mM tris (pH 7). About 500 µl of 1 mg/ml of each of the following samples was injected onto the 19-mer peptide column (4.6 mm D x 50 mm L): 1) pure IgGs from either human or mouse or chicken, 2) IgA (whole molecule), 3) human serum, 4) fetal bovine serum (1:10 dilution), or 5) 500 µl of 1 mg/ml mouse Igc, Fc fragment. After injection, the column was washed with 20 CVs of a mixture of 20mM tris buffer and 0.4 M NaCl (pH 7). The remaining bound proteins were eluted with 20 CVs of 12 mM HCl. Experiments with control POROS™-NH_2 (no peptide) column were done similarly. The bound fractions were collected and analyzed by SDS-Polyacrylamide gel electrophoresis.

Page 75, lines 2-7

Both solution phase and solid phase peptide(s) screening of mouse IgG (whole fragment) with the natural peptide library yielded one peptide of mass 1633 daltons and amino acid sequence CAQCHTVEK (SEQ ID NO: 8). Database search revealed that this peptide is a tryptic digest of cytochrome c (one of the proteins in the library) with a heme group covalently attached to the two cysteines at amino acid positions 14 and 17 of the protein.

Page 75, line 8 to page 76, line 3

The CAQCHTVEK (SEQ ID NO: 8) peptide with heme group was immobilized on POROS™AL® (aldehyde) via the N-terminal end. This POROS™ -peptide conjugate was used to separate IgG from serum at pH 8 under a 0-1M NaCl gradient. At pH 8, IgG was purified with comparable purity to that of IgG separated on POROS™ -Protein A column. The capacity of the POROS™ -peptide column was determined to be 10 mg/ml column volume which is comparable

to the binding capacity of POROS™-Protein A column. To determine the nature of interaction, the purification profile of IgG separated on the POROS™ -peptide column was compared with the IgG purified on standard ion exchange columns such as POROS™ -CM and POROS™ HQ. Results indicated that, under similar conditions, peptide columns exhibit predominantly ion exchange characteristics with secondary hydrophobic interactions and have a higher selectivity for IgG from serum than either of the ion exchange columns. The effect of the varying loading densities of peptide (from 10 mg/g POROS™ to 100 mg/g POROS™) on specificity and capacity for IgG binding was also investigated. The specificity of the peptide for IgG binding was also investigated. The specificity of the peptide for IgG did not vary with varying ligand densities, but the nature of interaction of IgG varied. At low loading density (10 mg/g POROS™) IgG bound primarily via ionic interaction requiring elution of bound IgG with salt gradient. At higher loading density (100 mg/mg) IgG bound strongly and eluted with acid buffer. The binding capacity varied from 1-2 mg/ml column volume at lower ligand density to 30 mg/ml column volume at higher ligand densities. The heme peptide POROS™ bound very weakly to HSA and only under very hydrophobic conditions (200 mM sodium sulphate, pH 7 buffer).

Page 76, lines 7-14

A simplified analog of the heme peptide (GAQGHTVEK) (SEQ ID NO: 9) was synthesized and immobilized on POROS™ AL via the N-terminal end. At pH 8, this GAQGHTVEK (SEQ ID NO: 9)-POROS™ conjugate bound specifically, and with comparable purity, to the IgG purified from human serum on POROS™ -Protein A column. The bound IgG was eluted from the GAQGHTVEK (SEQ ID NO: 9)-POROS™ column with 100 mM NaCl. The IgG binding capacity was determined to be 5 mg/ml column volume. Loading densities from 20 mg/g to 40 mg peptide /g POROS™ were evaluated. At these ligand densities, the specificity of IgG binding was not affected, but the capacity was reduced.

Page 76, line 17 to page 77, line 2

The Fc binding domain of recombinant proteins A and G and the amino acids involved in the binding of protein A to IgG have been mapped by site-directed mutagenesis (Fahnestock,

S.R., Alexander, P. Nagle, J. and Filpula, D., *J. Bacter* (1986) *167*(3):870-880). However, there has been no report of peptides isolated from these bacterial proteins that bind to IgG. By solution phase peptide(s) screening with native and denatured recombinant protein A and G digests against mouse IgG (Fc fragment) four peptides with remarkable overlapping sequences were identified. The peptide(s) were as follows: TVTEKPE (SEQ ID NO: 10), EKEPEVID (SEQ ID NO: 11), GDAPTPEKEPEASI (SEQ ID NO: 12) and TVTEKPEVIDASELTPAVT (SEQ ID NO: 7). The sequences of the larger peptides correlated with mass spec data. None of these peptides are typical tryptic digests, indicating that these peptides probably were selected from the native protein G digest. Database search revealed that all of these peptides were from recombinant Protein G. Further, the TVTE (SEQ ID NO: 13) sequence is a part of the Fc binding domain of recombinant protein G.

Page 77, lines 3-11

The TVTEKPEV (SEQ ID NO: 14) peptide was synthesized and immobilized on POROS™ via the N-terminal end. This POROS™ -TVTEKPEV (SEQ ID NO: 14) was found to bind IgG from human serum at pH 8 with comparable specificity as the POROS™-Protein A conjugate. The bound protein was eluted with 0-1M NaCl gradient. The TVTEKPEVIDASELTPAVT (SEQ ID NO: 7) peptide was synthesized directly on POROS™ - NH_2 resin via the C-terminal end. This peptide bound mouse IgG, Fc fragment with low capacity but high selectivity. The bound IgG was eluted with acid buffer. This peptide was more selective for IgG than IgA. Human IgG bound more selectively to the 19-mer peptide column that IgG from rabbit, goat or mouse.

Page 77, lines 12-19

One of the important and novel features is that from proteins A & G known to bind IgG with high affinity and requiring acidic conditions to elute the bound IgG, peptides were isolated, at least one of which (TVTEK) (SEQ ID NO: 15) has been shown to bind IgG, but with less affinity requiring only salt gradient for elution of the bound protein. Second, the peptide(s) of varying affinities and selectivity towards IgGs from different species have been identified. Third,

it is remarkable that two of the peptides namely, TVTEKPEVIDASELTPAVT (SEQ ID NO: 7) and TVTEKPEV (SEQ ID NO: 14) are part of the Fc binding domain of recombinant protein G.

Page 78, lines 2-23

Polyclonal antibodies are an interesting and logical source of peptides since they have specific antigen binding sites. A synthetic antibody fragment against lysozyme has been used as a ligand in immunoaffinity chromatography. This fragment was generated by molecular modeling of lysozyme and its antibody (Welling, G.W. *et al.*, (1990) *J. Chrom., 512*:337-343). Single chain antibodies that bind with weak affinities have also been generated against many targets by phage display (Griffiths, A.D. *et al.*, (1994) *The EMBO J., 13*(14):3245-3260). To date there has been no report of selection of target specific peptide(s) isolated from polyclonal antibody digests. Tryoptic digests of denatured anti-IgG (Fc specific) polyclonal antibodies raised in rabbit, goat and sheep were run through a POROS™ epoxy column immobilized with IgG. The bound peptides were eluted onto an RP column and characterized. The amino acid sequence was determined to be GAQGHTVEK (SEQ ID NO: 9). A database search revealed that the GAQGHTVEK (SEQ ID NO: 9) sequence is a part of the variable region of the light chain of IgG. Note that the HTVEK (SEQ ID NO: 16) motif is also found in the heme peptide of cytochrome c. The heme peptide has been shown, as above, to bind IgG. Additionally, the TVEK (SEQ ID NO: 17) motif is similar to the TVTEK (SEQ ID NO: 15) sequence found in the IgM heavy chain, T-cell receptor (beta chain) and also IgG binding proteins such as protein G and protein LG. Protein LG, a hybrid molecule of protein L and G, binds to intact IgGs, as well as Fc and Fab fragments and IgG light chains. The characteristics of GAQGHTVEK (SEQ ID NO: 9) peptide as affinity surface for IgG binding have been discussed above. The most important and novel feature is that from a mixture of denatured antibodies, one peptide was isolated that was selective for IgG.

To the Claims

Please cancel without prejudice claims 1-48.

Please add new claims 49-66 as follows.

49. (New) A method of analyzing the relative binding affinities of at least a first ligand and a second ligand to a target using a tandem column chromatography system, the method comprising the steps of:

(a) providing a solution comprising a plurality of mixed ligands and a target, wherein the plurality of mixed ligands comprises at least a first ligand and a second ligand;

(b) introducing a sample of the solution to a first column, the first column comprising a size exclusion medium;

(c) passing a number of column volumes of a solvent free of the target and ligands which bind to the target through the first column at a linear velocity;

(d) introducing an early portion of the eluant from the first column directly into a second column to determine the presence or absence of the first ligand and the second ligand;

(e) introducing another sample of the solution to the first column;

(f) passing a number of column volumes of a solvent free of the target and ligands which bind to the target through the first column at another linear velocity;

(g) introducing an early portion of the eluant from the first column directly into the second column to determine the presence or absence of the first ligand and the second ligand; and

(h) determining the relative binding affinities of at least the first ligand and the second ligand for the target based on the results of steps (d) and (g), where the presence of one of the first ligand and the second ligand at a decreased linear fluid velocity correlates to a higher relative binding affinity for that ligand to the target.

50. (New) The method of claim 49 further comprising repeating steps (e)-(g) a desired number of times.

51. (New) The method of claim 49 further comprising the step of identifying at least one of the first ligand or the second ligand.

52. (New) The method of claim 51 wherein the step of identifying comprises using mass spectrometry.

53. (New) The method of claim 49 wherein the second column is an affinity chromatography column.

54. (New) The method of claim 49 wherein the second column is a ligand accumulator.

55. (New) The method of claim 49 wherein the first ligand is selected from the group consisting of a protein, a peptide, a polysaccharide, and a polynucleotide.

56. (New) The method of claim 51 further comprising the step of synthesizing an identified ligand.

57. (New) A method of determining the presence of a ligand having a relatively high on-rate for a target in a solution of mixed ligands, the method comprising the steps of:

introducing a solution comprising a target and mixed ligands to a first column comprising a size exclusion medium, wherein at least a first ligand of the mixed ligands forms a first complex with the target; and

eluting the first column with a solvent free of mixed ligands and the target at various linear fluid velocities to modulate the binding selectively of at least the first ligand to the target in preference to other mixed ligands,

wherein at various linear fluid velocities early outputs comprising the first complex from the first column are indicative of the first ligand having a relatively high on-rate for the target compared to other mixed ligands in the solution.

58. (New) The method of claim 57 comprising the additional steps of eluting an early portion of the output of the first column directly into a second column and eluting the second column to determine the presence of the first ligand.

59. (New) The method of claim 58 wherein the second column is an affinity chromatography column.

60. (New) The method of claim 58 wherein the second column is a ligand accumulator.

61. (New) The method of claim 57 further comprising the step of identifying the first ligand.

62. (New) The method of claim 61 wherein the step of identifying comprises using mass spectrometry.

63. (New) The method of claim 58 further comprising the step of identifying the first ligand.

64. (New) The method of claim 63 wherein the step of identifying comprises using mass spectrometry.

65. (New) The method of claim 61 further comprising the step of synthesizing the first ligand.

66. (New) The method of claim 57 wherein the first ligand is selected from the group consisting of a protein, a peptide, a polysaccharide, and a polynucleotide.

REMARKS

Claims 1-48 are pending. Applicants cancel without prejudice claims 1-48. Applicants introduce new claims 49-66 for examination. Consequently, claims 49-66 are pending after entry of this Amendment. Applicants respectfully submit that claims 49-66 introduce no new matter and are in condition for allowance.

To The Specification

The specification is amended to include a specific reference to earlier filed applications for which the benefit of their filing dates is claimed under 35 U.S.C. § 120.

The specification is amended to comply with the sequence rules set forth in 37 C.F.R. 1.821 through 1.825. That is, the proper sequence identifiers are inserted after the appropriate sequence.

The specification is also amended in five places to replace erroneous citations of the 19-mer peptide TVTEKPEVIDASELTPAVT (SEQ ID NO: 7). The following recitations of the 19-mer peptide were amended to the correct peptide sequence: TVTEKVIDASELTPAVT on page 73, line 3; TVTEKPEVIDASELPAVT on page 74, line 2; TVTEKPEVIDASELPAVT on page 76, line 26; TVETEKPEVIDASELTPAVT on page 77, line 8; and TVTEKEPEVIDASELTPAVT on page 77, line 19.

Support for these amendments is found in U.S. Provisional Patent Application Serial No. 60/000,518, to which the instant application claims the benefit of, through U.S. Serial Nos. 09/267,993 and 08/670,670, and the complete disclosure of which was incorporated by reference into U.S. Serial Nos. 09/267,993 and 08/670,670 and the instant application. Specifically, the proper sequence of the 19-mer peptide is found in U.S. Serial No. 60/000,518 on page 13, line 13, referring to Figure 17.

Accordingly, Applicants submit that these amendments introduce no new matter.

New Claims

New claims 49-66 relate to embodiments of methods of the invention which use a column including a size exclusion medium to separate ligands which bind to a target from unbound ligands. More specifically, new independent claim 49 relates to a method of analyzing the relative binding affinities of at least a first ligand and a second ligand to a target using tandem

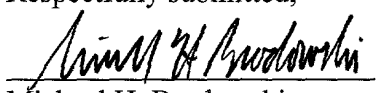
column chromatography. New independent claim 57 relates to a method of determining the presence of a ligand having a relatively high on-rate for a target in a solution of mixed ligands.

Support for new claims 49-66 is found in the application as filed at least at page 13, definition of "complex;" page 18, lines 1-2; page 23, line 6 to page 24, lines 21; page 28, line 23 to page 29, line 7; page 30, lines 7-15; page 32, lines 3-5; page 34, lines 6-11; page 35, lines 1-3; page 38, line 12 to page 39, line 10; and in originally filed claims 21-23, 35 and 36. Accordingly, Applicants submit that no new matter is added by the new claims.

CONCLUSION

In view of the new claims and remarks submitted herein, Applicants respectfully submit that claims 49-66 are in condition for allowance and request the application proceed to issue. The Examiner is invited to call the undersigned if the Examiner believes that a telephone conversation could be helpful in expediting prosecution of the instant application.

Respectfully submitted,



Dated: December 5, 2001

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MARKED-UP VERSION OF AMENDED PARAGRAPHS OF SPECIFICATION

Page 1, lines 9-12

RELATED APPLICATIONS [~~APPLICATION~~]

This application is a divisional of application Serial No. 09/267,993, filed on March 12, 1999, which is a continuation of Serial No. 08/670,670, filed on June 26, 1996, now abandoned, which claims the benefit of [~~is based on provisional application serial number~~] Serial No. 60/000,518, filed on June 26, 1995, now expired, the entire disclosures of which are incorporated by reference herein [~~disclosure of which in its entirety is herein incorporated by reference~~].

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Page 59, lines 2-14

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Page 62, line 20 to page 63, line 19

Furthermore, increasing the number of column volumes used to wash column 1 prior to

elution onto column 2 resulted in a decrease in the subsequent recovery of these molecules, presumably due to dissociation of these agents from the LPS while on column 1. The number of wash volumes required to reduce the peak for these agents correlated with the reported affinities of these agents for LPS; PmxB (Kd 0.4 mM) was reduced 50% after 42 CVs washing of column 1 while pentamidine (Kd 100 nM) required a higher number of washes for a similar reduction (46 CVs). Using this methodology we could rapidly examine which peaks from the library were capable of binding to LPS with the highest affinity since, upon exposure to higher numbers of wash volumes only the peptides with higher affinity for the target were retained. The relative reduction in the peak area (or height) for each peak eluted from the reverse phase column when plotted against the number of CVs used to wash column 1 could be used to distinguish the peaks with higher affinity since these showed a lower rate of reduction under the same conditions. Three such peaks from the XXXFL library and 3 peaks from the CXXXC library were further purified and characterized by MS and peptide sequencing. Results from the sequencing data for the XXXFL candidates suggested the structures RRRFL (SEQ ID NO: 3), RRKFL (SEQ ID NO: 4) or KKRFL (SEQ ID NO: 5). The latter peptide has been synthesized and demonstrated to bind to LPS using the same paradigm above. However, while this peptide is displaced by Pentamidine in a competition experiment, its affinity for LPS is much lower than the latter molecule. The other peptides will be synthesized and studied in a similar manner although another approach being considered is that a sublibrary of the format ZZZFL (where Z represents either R or K) be screened to identify the member with the highest affinity. Candidates purified from the cyclic library are still awaiting sequencing.

Page 66, line 25 to page 67, line 6

Figure 8 shows the results of this data. From this data we 'named' the peptide HHRSY (SEQ ID NO: 6) as being composed of the amino acids that showed the greatest increase in AA% when the column lacked sugar relative to its AA% in the presence of sugar. Synthesis of this peptide and characterization of its ability to bind revealed that it was capable of binding specifically to Con A immobilized on the column with little binding to the control column. Furthermore, this peptide could be displaced somewhat by inclusion of a high concentration of the competing sugar ligand. These data suggest that it is possible to identify ligands for a

specific site on a molecule by using this bimolecular approach to screening.

Page 73, lines 2-11

2) Direct synthesis of peptides on POROS™: The 19-mer peptide [~~(TVTEKVIDASELTPAVT)~~] (TVTEKPEVIDASELTPAVT) (SEQ ID NO: 7) selected from protein A and G digests was directly synthesized on 20 μm amine-functionalized ™ particles by standard FMOC chemistry. About 700 mg of resin was dry packed into a 4.6 mm D x 50 mm L PEEK column equipped with 2 μm frits. The packed column was attached with appropriate adapters to a PerSeptive Biosystems 9050 plus continuous flow peptide synthesizer. Upon completion of synthesis, the resin was dried and deprotected using 95% TFA/5% triisopropylsilane for 24hrs. The final peptide-support conjugate was packed using POROS™ Self Pack® column packing device at flow rate 10 ml/min onto 4.6 mm D x 50 mm L column for evaluation as affinity supports.

Page 74, lines 1-11

3) Purification of IgG (whole and Fc fragment) on POROS™-peptide [~~(TVTEKPEVIDASELPAVT)~~] (TVTEKPEVIDASELTPAVT) (SEQ ID NO: 7) column: The 19-mer peptide column (4.6 mm D x 50 mm L) was equilibrated with 20 CVs of equilibration buffer, 20 mM tris (pH 7). About 500 μl of 1 mg/ml of each of the following samples was injected onto the 19-mer peptide column (4.6 mm D x 50 mm L): 1) pure IgGs from either human or mouse or chicken, 2) IgA (whole molecule), 3) human serum, 4) fetal bovine serum (1:10 dilution), or 5) 500 μl of 1 mg/ml mouse Igc, Fc fragment. After injection, the column was washed with 20 CVs of a mixture of 20mM tris buffer and 0.4 M NaCl (pH 7). The remaining bound proteins were eluted with 20 CVs of 12 mM HCl. Experiments with control POROS™-NH_2 (no peptide) column were done similarly. The bound fractions were collected and analyzed by SDS-Polyacrylamide gel electrophoresis.

Page 75, lines 2-7

Both solution phase and solid phase peptide(s) screening of mouse IgG (whole fragment)

with the natural peptide library yielded one peptide of mass 1633 daltons and amino acid sequence CAQCHTVEK (SEQ ID NO: 8). Database search revealed that this peptide is a tryptic digest of cytochrome c (one of the proteins in the library) with a heme group covalently attached to the two cysteines at amino acid positions 14 and 17 of the protein.

Page 75, line 8 to page 76, line 3

The CAQCHTVEK (SEQ ID NO: 8) peptide with heme group was immobilized on POROS™AL® (aldehyde) via the N-terminal end. This POROS™ -peptide conjugate was used to separate IgG from serum at pH 8 under a 0-1M NaCl gradient. At pH 8, IgG was purified with comparable purity to that of IgG separated on POROS™ -Protein A column. The capacity of the POROS™ -peptide column was determined to be 10 mg/ml column volume which is comparable to the binding capacity of POROS™-Protein A column. To determine the nature of interaction, the purification profile of IgG separated on the POROS™ -peptide column was compared with the IgG purified on standard ion exchange columns such as POROS™ -CM and POROS™ HQ. Results indicated that, under similar conditions, peptide columns exhibit predominantly ion exchange characteristics with secondary hydrophobic interactions and have a higher selectivity for IgG from serum than either of the ion exchange columns. The effect of the varying loading densities of peptide (from 10 mg/g POROS™ to 100 mg/g POROS™) on specificity and capacity for IgG binding was also investigated. The specificity of the peptide for IgG binding was also investigated. The specificity of the peptide for IgG did not vary with varying ligand densities, but the nature of interaction of IgG varied. At low loading density (10 mg/g POROS™) IgG bound primarily via ionic interaction requiring elution of bound IgG with salt gradient. At higher loading density (100 mg/mg) IgG bound strongly and eluted with acid buffer. The binding capacity varied from 1-2 mg/ml column volume at lower ligand density to 30 mg/ml column volume at higher ligand densities. The heme peptide POROS™ bound very weakly to HSA and only under very hydrophobic conditions (200 mM sodium sulphate, pH 7 buffer).

Page 76, lines 7-14

A simplified analog of the heme peptide (GAQGHTVEK) (SEQ ID NO: 9) was

synthesized and immobilized on POROS™ AL via the N-terminal end. At pH 8, this GAQGHTVEK (SEQ ID NO: 9)-POROS™ conjugate bound specifically, and with comparable purity, to the IgG purified from human serum on POROS™ -Protein A column. The bound IgG was eluted from the GAQGHTVEK (SEQ ID NO: 9)-POROS™ column with 100 mM NaCl. The IgG binding capacity was determined to be 5 mg/ml column volume. Loading densities from 20 mg/g to 40 mg peptide /g POROS™ were evaluated. At these ligand densities, the specificity of IgG binding was not affected, but the capacity was reduced.

Page 76, line 17 to page 77, line 2

The Fc binding domain of recombinant proteins A and G and the amino acids involved in the binding of protein A to IgG have been mapped by site-directed mutagenesis (Fahnestock, S.R., Alexander, P. Nagle, J. and Filpula, D., *J. Bacter* (1986) *167*(3):870-880). However, there has been no report of peptides isolated from these bacterial proteins that bind to IgG. By solution phase peptide(s) screening with native and denatured recombinant protein A and G digests against mouse IgG (Fc fragment) four peptides with remarkable overlapping sequences were identified. The peptide(s) were as follows: TVTEKPE (SEQ ID NO: 10), EKEPEVID (SEQ ID NO: 11), GDAPTPEKEPEASI (SEQ ID NO: 12) and TVTEKPEVIDASELTPAVT (SEQ ID NO: 7) [~~TVTEKPEVIDASELPAVT~~]. The sequences of the larger peptides correlated with mass spec data. None of these peptides are typical tryptic digests, indicating that these peptides probably were selected from the native protein G digest. Database search revealed that all of these peptides were from recombinant Protein G. Further, the TVTE (SEQ ID NO: 13) sequence is a part of the Fc binding domain of recombinant protein G.

Page 77, lines 3-11

The TVTEKPEV (SEQ ID NO: 14) peptide was synthesized and immobilized on POROS™ via the N-terminal end. This POROS™ -TVTEKPEV (SEQ ID NO: 14) was found to bind IgG from human serum at pH 8 with comparable specificity as the POROS™-Protein A conjugate. The bound protein was eluted with 0-1M NaCl gradient. The TVTEKPEVIDASELTPAVT (SEQ ID NO: 7) [~~TVETEKPEVIDASELTPAVT~~] peptide was

synthesized directly on POROS™ -NH_2 resin via the C-terminal end. This peptide bound mouse IgG, Fc fragment with low capacity but high selectivity. The bound IgG was eluted with acid buffer. This peptide was more selective for IgG than IgA. Human IgG bound more selectively to the 19-mer peptide column that IgG from rabbit, goat or mouse.

Page 77, lines 12-19

One of the important and novel features is that from proteins A & G known to bind IgG with high affinity and requiring acidic conditions to elute the bound IgG, peptides were isolated, at least one of which (TVTEK) (SEQ ID NO: 15) has been shown to bind IgG, but with less affinity requiring only salt gradient for elution of the bound protein. Second, the peptide(s) of varying affinities and selectivity towards IgGs from different species have been identified. Third, it is remarkable that two of the peptides namely, [~~TVTEKEPEVIDASELTPAVT~~] TVTEKPEVIDASELTPAVT (SEQ ID NO: 7) and TVTEKPEV (SEQ ID NO: 14) are part of the Fc binding domain of recombinant protein G.

Page 78, lines 2-23

Polyclonal antibodies are an interesting and logical source of peptides since they have specific antigen binding sites. A synthetic antibody fragment against lysozyme has been used as a ligand in immunoaffinity chromatography. This fragment was generated by molecular modeling of lysozyme and its antibody (Welling, G.W. *et al.,* (1990) *J. Chrom., 512*:337-343). Single chain antibodies that bind with weak affinities have also been generated against many targets by phage display (Griffiths, A.D. *et al.,* (1994) *The EMBO J., 13*(14):3245-3260). To date there has been no report of selection of target specific peptide(s) isolated from polyclonal antibody digests. Tryoptic digests of denatured anti-IgG (Fc specific) polyclonal antibodies raised in rabbit, goat and sheep were run through a POROS™ epoxy column immobilized with IgG. The bound peptides were eluted onto an RP column and characterized. The amino acid sequence was determined to be GAQGHTVEK (SEQ ID NO: 9). A database search revealed that the GAQGHTVEK (SEQ ID NO: 9) sequence is a part of the variable region of the light chain of IgG. Note that the HTVEK (SEQ ID NO: 16) motif is also found in the heme peptide of cytochrome c. The heme peptide has been shown, as above, to bind IgG. Additionally, the

TVEK (SEQ ID NO: 17) motif is similar to the TVTEK (SEQ ID NO: 15) sequence found in the IgM heavy chain, T-cell receptor (beta chain) and also IgG binding proteins such as protein G and protein LG. Protein LG, a hybrid molecule of protein L and G, binds to intact IgGs, as well as Fc and Fab fragments and IgG light chains. The characteristics of GAQGHTVEK (SEQ ID NO: 9) peptide as affinity surface for IgG binding have been discussed above. The most important and novel feature is that from a mixture of denatured antibodies, one peptide was isolated that was selective for IgG.